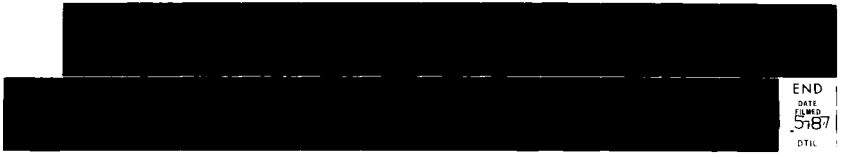
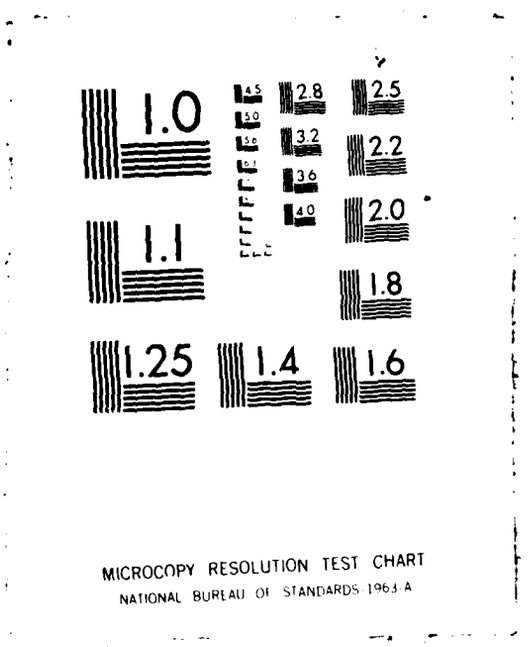
AD-A178 513 ELECTRICALLY MEDIATED TRAUMA REPAIR(U) PURDUE UNIV LAFAYETTE IN R B BORGENS MAR 86 DAMD17-84-C-4012 1/1

UNCLASSIFIED F/G 6/16 NL



END
DATE FILMED
5-87
DTIC


1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0
5.6
6.3
MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS 1963-A

AD_____

ELECTRICALLY MEDIATED TRAUMA REPAIR

Annual and Final Report

Richard B. Borgens
Associate Professor

March 1986

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-84-C-4012

Purdue University
West Lafayette, Indiana 47907

DOD DISTRIBUTION STATEMENT

Approved for public release; distribution unlimited

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release: distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: Purdue University; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): West Lafayette, IN 47907	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research and Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-84-C-4012
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, MD 21701-5012	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 61102A; PROJECT NO. 3M161102BS10; TASK NO. BA; WORK UNIT ACCESSION NO. 450

11. TITLE (Include Security Classification)
Electrically Mediated Trauma Repair (U)

12. PERSONAL AUTHOR(S)
Richard B. Borgens, Ph.D.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual/Final*	FROM 10/1/83 TO 11/30/85	March 1986	

16. SUPPLEMENTARY NOTATION
*Annual for the period 1 Oct 84 - 30 Nov 85
Final for the period 1 Oct 83 - 30 Nov 85

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	01	
06	05	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
spinal cord; regeneration; bone; bioelectricity

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Our laboratory has studied the role that a naturally produced ionic current plays in animal development and regeneration. Such steady currents and voltage gradients are produced by physiological batteries in the integument, the inner investments of bone, or the cell membrane. We have developed means to modify these currents in order to test their relevance to certain developmental (such as limb development) or regeneration (such as fracture repair and axonal elongation). In some cases we can modify the battery driving these currents by modifying the ionic composition of the bathing fluid - in other cases, we can directly alter the

(Continued on reverse)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT: ☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION: Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL: Mrs. Judy Pawlus	22b. TELEPHONE (Include Area Code): 301/663-7325; 22c. OFFICE SYMBOL: SGRD-RMI-S

DD Form 1473, JUN 86 Previous editions are obsolete. SECURITY CLASSIFICATION OF THIS PAGE

character of the fields by electronic means. This latter ability has provided techniques that bear medical import - which is the basic thrust of the research supported by the Department of the Army. We have concentrated on two areas: fracture repair and ways to electically modify it, and central nervous system regeneration - especially spinal cord regeneration.

In bone we have provided the first measurements of a natural current of injury entering the fracture gap. This current is similar in density and polarity to the currents used in the clinic to induce healing in chronic fracture non-unions. We have applied currents ~~of a similar character~~ to intact bone and have found that we can grossly alter the shape - or more properly - alter the remodeling of hard tissue. Now we wish to test if such current can increase the rate of normal fracture repair.

Using a novel analytical technique, we have demonstrated that an applied electric field is able to induce the regeneration of axons of the dorsal columns in adult guinea pigs. This is the first demonstration of this kind in the literature, and one of only three extant techniques to induce linear elongation in long-tract central neurons. Moreover, our technique is non-invasive and does not require surgical manipulation of the spinal cord. We are now testing if this induced regeneration is coincident with functional recovery. Ultimately, we hope these techniques provide novel clinical tools. They would have special relevance to the military in providing more rapid clinical solutions to these intractable lesions of hard tissue and of neurons - the most severe and common lesions observed in combat casualities or injuries sustained during military training.

A-1

ABSTRACT

Our laboratory has studied the role that a naturally produced ionic current plays in animal development and regeneration. Such steady currents and voltage gradients are produced by physiological batteries in the integument, the inner investiments of bone, or the cell membrane. We have developed means to modify these currents in order to test their relevance to certain developmental pageants (such as limb development) or regeneration (such as fracture repair and axonal elongation). In some cases we can modify the battery driving these currents by modifying the ionic composition of the bathing fluid - in other cases, we can directly alter the character of the fields by electronic means. This latter ability has provided techniques that bear medical import - which is the basic thrust of the research supported by the Department of the Army. We have concentrated on two areas: fracture repair and ways to electrically modify it, and central nervous system regeneration - especially spinal cord regeneration.

In bone we have provided the first measurements of a natural current of injury entering the fracture gap. This current is similar in density and polarity to the currents used in the clinic to induce healing in chronic fracture non-unions. We have applied currents of a similar character to intact bone and have found that we can grossly alter the shape - or more properly - alter the remodeling of hard tissue. Now we wish to test if such current can increase the rate of normal fracture repair.

Using a novel analytical technique, we have demonstrated that an applied electric field is able to induce the regeneration of axons of the dorsal columns in adult guinea pigs. This is the first demonstration of this kind in the literature, and one of only three extant techniques to induce linear elongation in long-tract central neurons. Moreover, our technique is non-invasive and does not require surgical manipulation of the spinal cord. We are now testing if this induced regeneration is coincident with functional recovery. Ultimately, we hope these techniques provide novel clinical tools. They would have special relevance to the military in providing more rapid clinical solutions to these intractable lesions of hard tissue and of neurons - the most severe and common lesions observed in combat casualities or injuries sustained during military training.

AD_____

ELECTRICALLY MEDIATED TRAUMA REPAIR

Annual and Final Report

Richard B. Borgens
Associate Professor

March 1986

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD17-84-C-4012

Purdue University
West Lafayette, Indiana 47907

FOREWORD

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.

TABLE OF CONTENTS

TABLE OF CONTENTS

A. Statement of Problem; Annual and Final

For many years we have studied the role of naturally produced voltage gradients in the development and regeneration of animals. We have developed special instrumentation to accomplish this in a very precise way (see below). One approach to testing the relevance of these natural currents and voltages is to modify them in predictable and reproducible ways and in so doing modify development. The various systems that we have applied this conceptual approach to includes amphibian limb regeneration and limb development, the regeneration of eyes in gastropod molluscs, the regeneration of reticulospinal neurons in the lamprey central nervous system, fracture healing, integumentary wound healing, and CNS regeneration in the mammal. The bulk of the work supported by the DOD contract (Department of the Army) involved studies of bone and nerve regeneration in the mammal.

In bone we were interested in knowing if fractures do drive intense currents of injury through the lesion. This would provide a rationale for the clinical use of electricity in healing chronic fracture nonunions as well as provide a physiological basis for improving the clinical use of current (for example, can one modify intact bone in a predictable way with applied currents and can one modify longstanding injuries by reshaping bone with applied fields?). During this contract period we have answered the first question and have preliminary answers to the others. In mammalian CNS regeneration our first problem was to develop an unambiguous technique for: 1) applying currents across the hemisected spinal cord of the mammal; 2) analyzing axonal regeneration within the cord (single fiber analysis); and 3) developing a marker for exact plane of the injury so that one can analyze the cord months after an injury and know how to discriminate lesioned from surviving or even unlesioned fibers. Our second problem was to apply these techniques to severed spinal cords of guinea pigs and to determine if an applied electric field will induce regeneration. During this contract period we have answered all of these experimental questions.

B. Background; Annual and Final

1. Bone Bioelectricity

Mammalian bone is a dynamic tissue. It modulates its shape in response to changes in load, and possesses the ability to heal itself spontaneously. A variety of medical conditions (congenital pseudoarthrosis, diabetes, and chronic osteomylitis are but just a few) are known to effect the healing of bone fractures leading to a clinical syndrome called the chronic nonunion. Such nonunions can be intractable lesions, failing numerous bone-grafts, and sometimes as a last resort require amputation of the affected extremity. In the last decade one novel treatment for such nonunions has been developed that apparently provides a high degree of success in producing a stable union. This clinical treatment, pioneered by Carl Brighton at the University of Pennsylvania Medical School, is the application of a steady, direct electric current to the fracture gap (1).

When electric current is artificially pulled into the fracture gap by an implanted cathodic (negative) electrode for several months, the clinical success rate approached 80% (1). The magnitude of this current is usually on the order of 10's of microamperes of total current; and the necessary polarity is negative within the lesion (2). The positive (anodal) electrode

can be placed either on the skin's surface or subdermally. It is reasonable to ask why such an unorthodox treatment is effective at all. This cannot be answered because we have little (if any) knowledge of the basic physiological principles underlying these clinical observations since we have no measurement of an endogenous current in living bone reported in the literature. Such a current could provide a rationale for the effectiveness of applied currents in the clinic.

It is well known that bone produces transient voltages in response to stress (3 and 4). Steady voltages also exist along intact living bone and in or near fractures (5,6). These surface-detected voltages provide little quantitative data for physiologists and have in most cases not been measured under physiological conditions. Freindenberg and Brighton (5) found such "bioelectric potentials" to be negative in articular and epiphyseal regions and in the fracture site. Conversely, Becker reported a "positive" shift in injury potential at the fracture site (suggesting current leaving the injury) and a positive shift after periosteal injury (7). Injured muscle has also been suggested as the source of "fracture potentials". Considering the above, it is obvious that the plethora of techniques applied to bone have not produced a coherent picture of voltage changes in dead specimens, much less living bone.

Transient changes in voltage have been ascribed to both piezoelectricity, and to streaming potentials. It is unquestioned that tendon, bone, collagen, and various other well-ordered biological materials exhibit piezoelectric properties in the dry state. However, recent experimentation greatly favors the streaming potential as the dominant mechanism underlying load-induced short-lived potentials in physiologically wet bone (8).

It has become widely accepted (without rigorous experimental support) that bone remodeling (Wolff's law) is dependent upon load-induced voltages. Bioelectric measurements suggested that the compression side of flexed bone is electro-negative with respect to the tension side. "Negative potentials" during flexure are said to generate bone deposition, and "positive potentials" are said to be responsible for bone resorption. This notion has also been applied to the adaptive remodeling that occurs after a fracture (8). Once current enters the bone substance, its pathway and densities are entirely unknown. Bone is very porous, with many canals and lucanae that interconnect with each other. The overall resistances to the flow of current would be inversely proportional to the extracellular space within these portals. Thus, it would be difficult to speculate on the magnitude of the fields associated with current flow. The electrical fields pertinent to biological activity would be those voltage gradients imposed on living cells existing along this current path. We set out to answer these questions using a vibrating probe for the determination of extracellular current (see below). This device allows a precise determination of current and voltage in <u>living</u> specimens immersed in a natural medium. It does so non-invasively. Thus, we hoped to learn the true nature of bone bioelectricity and how to tie this knowledge into the biophysics of orthopedic medicine.

2. <u>Applied Electric Fields and Nerve Regeneration</u>

Sven Ingvar (9) was the first investigator to directly test the notion that an applied electric field may enhance nerve regeneration. There have been many classical experiments since this time, however, I will not review this

older literature on galvanotropism or galvanotaxis (7,10). The first carefully controlled and thoughtful test of such ideas were conducted by Marsh and Beams in 1946.(11) They observed a heightened growth response (and a guided movement) of neurites emanating from explanted chick DRG toward the negative pole of an applied electrical field. Paul Weiss (12) criticized all such experiments suggesting that applied fields orient molecules in the culture substrate and this explained the "directional responses" of neurites exposed to artificially applied electricity. This single opinion held for many years, and until more modern times, the entire area was held to be controversial, and the responses of neurons to artificially applied fields as either artifactual or due to secondary variables independent of the field.

Jaffe and Poo (10) notes that Weiss's arguments were unsound because modern studies of electricially induced birefrigence alterations in well-ordered macro molecules (such as collagen) demonstrate that 100's to 1000's of volts cm^{-1} were necessary to achieve realignment or reordering. Nerves in culture responded to 10-100 mV/mm. They repeated Marsh and Beam's seminal study (using chick DRG in culture) and confirmed most of the previous investigators findings (except they noted no directional responses by neurites). Moreover, they used markers in the substrate to monitor movement of the explanted ganglion (in addition to the neurites). They discovered that the ganglion mass has a proclivity to migrate toward the anode (+ pole of the applied field). If neurites facing the opposite pole (- pole) are "stuck" to the substrate at their tips, then they can be stretched as the ganglion moves toward the anode. This gives the <u>impression</u> of neurite elongation toward the antipode (cathode). When careful marking techniques are employed the results confirmed that still a significant (even luxurious) growth of neurites is stimulated facing the negative (-) pole of the field imposed across the culture. Other groups pursuing these studies still do not focus their attention on ganglionic movement, thus most other experiments by other modern groups using explanted ganglia are still ambiguous.

Recently, three more culture experiments provide unequivocal proof of electrically induced growth responses in nervous tissue. Hinkle et al. (13) and Patel and Poo (14,15) have observed the responses of individual differentiating neuroblast neurites in culture to applied fields. Individual cells are obtained from disaggregated Xenopus neurula stage embryos and such cells develop well in culture. Individual growing neurites will bend through great arcs to deviate their axis of growth toward the negative pole of the applied field. The rate and amount of neurite growth was enhanced in this vector, and always parallel with the long axis of the applied field. In the latter study, if the axis of the field was changed during the experiment, neurites (originally) growing toward the cathode reversed their direction of growth and reoriented themselves - growing toward the new position of the negative pole. These experiments were well controlled, fields were applied with salt bridges, and cells were grown on tissue culture plastic (no substrate was used). Taken together they constitute formal proof that a DC applied electric field can grossly influence nerve growth.

Over the years, our group has shown such responses can be achieved <u>in vivo</u> as well. We have shown that minute, steady electrical fields imposed within the forelimb stumps of adult frogs initiated limb regeneration. Furthermore, a striking <u>hyperinnervation</u> of such electrically treated limbs was observed both in <u>Rana</u> (Borgens et al. [16]) and in <u>Xenopus</u> (Borgens et al. [17]). This suggested to us that the induced regeneration was mediated by an

enhancement of nerve growth within the terminal portions of the limb stumps and thereby intitiated limb regeneration in the same manner as the famous surgical hyperinnervations of Rana stumps by Marcus Singer. However, it was quite clear that in response to current delivered to the tissues by wick electrodes (salt bridges) striking amounts of nerve regenerated within the new structure. In Rana, as much as 20% of the terminal portion of the regenerate was nerve tissue (determined by morphometric analysis of serial cross sections of stump and regenerate). In Xenopus, nerve trunks ramified throughout the cartilage core of the regenerate in all of the electrically treated stumps (Borgens et al. [17,28]). In sham-treated controls, it was very rare indeed to find any such nerve within the hypomorphic regenerates that form naturally (see Borgens et al. [18], reviewed by Borgens [7]).

Using long flexible saline bridges (or wick electrodes), we imposed an electric field (on the order of 10 mV/mm) across the completely severed spinal cord of lamprey larvae for 5 to 6 days, with the anode rostral and the cathode caudal to the lesion.(19) Sham-treated animals were treated identically to experimentals except that no current was delivered to the tissues. In our preliminary experiments, of the 15 current-treated and 15 sham-treated animals, 11 and 13, respectively, survived to provide data. At about 55 days post transection, we assayed the responses to these electrical applications by a combination of simultaneous extracellular and intracellular recording of Action Potential (AP) propagation across the lesion. After electrical records were taken (by antidromic and orthodromic stimulation and recording across the lesions), the axons responsible for the intracellular records were injected with the fluorescent dye Lucifer yellow. (The recording intracellular microelectrode was filled with this dye.) This allowed us to compare the anatomy of those descending reticulospinal neurons that propagated AP's across the lesion with those neurons that did not. I will summarize our main findings: (a) In 73% of the animals treated with electric current, APs elicited by extracellular stimulation of the whole spinal cord were propagated in both directions across the lesion. (b) In most (69%) of the sham-treated controls, APs did not propagate across the lesion in either direction. This is not surprising since only modest axonal regeneration is usually observed at 100-150 days post transection in the lamprey. We assayed at about 55 days post transection. This gave us a reasonably unambiguous baseline with which to compare the effects of our treatments. (c) Intracellular recording was combined with extracellular recording and fluorescent dye labeling of individual cells to characterize the giant axons responsible for propagating APs across the lesion. Axons that conducted spikes antidromically across the lesion site were found to traverse it, in a few cases, terminate within the lesion. Few axons terminating within, and no axons ending proximal to the lesion could be fired by stimulating distal to the lesion and recording just behind the brain. These tests demonstrate that the increased occurrence of APs porpagating across the lesion in electrically treated cords can be ascribed to the increased number of axons ending in or beyond the lesion area. (d) The greatest number of fluorescently labeled axons in current-treated cords were found within or through the lesion by about 55 days post transection. Most of a comparable population of axons in the sham-treated controls had ended proximal to the lesion. (e) The morphology of most of the terminal ends of identifiable giant axons found in experimentally treated spinal cords was indicative of actively growing regenerating fibers. Most of the ends of control fibers were relatively undifferentiated morphologically, appearing as axons that are in a less active growth state, perhaps even in stasis.

Altogether, we felt that these results encouraged further testing of the hypothesis that applied electrical fields may enhance the regeneration of CNS nerves in vertebrates.

Recent experiments (Roederer et al. [29]) have demonstrated that an early effect of the artificially applied field may be to reduce the amount of "dieback" or retrograde axonal degeneration that occurs after axonal transection. Since a large Ca^{++} and Na^{+} injury current is driven into the ends of lesioned nerves (note - an endogenous flow of current), the reasoning is that that applied field produces a bucking voltage reducing the probable destructive effects of the early endogenous current. We had suggested this as a mechanism of effect in our original description of such endogenous currents of injury in lamprey reticulospinal neurons (reviewed and discussed in Borgens et al. [7]). However, this view provides only partial insight into electrical field mediated responses in nerves. It does not speak to the fact that electrically enhanced regeneration in lamprey reticulospinal neurons is only apparent more than a month after transection (differences between controls and experimentals are insignificant prior to this time - Borgens et al. [19]); nor does it speak to field effects on intact, growing neurons (discussed above); nor does it explain directional responses at all.

I discuss this only to suggest that we have insights into putative mechanisms of effect. They are of fundamental importance, and an ongoing part of our laboratory's investigations (still using the lamprey system). I hope to have persuaded the reader that applied electrical fields can induce significant growth responses in damaged or developing vertebrate axons (by whatever mechanism), both in vivo and in vitro. In fact, a close comparison of the above mentioned culture experiments to similar such experiments testing the effects of Nerve Growth Factor (NGF) on axonal outgrowth and directionally suggests that certain responses (such as directional guidance) are quite profound when applied fields are used as an effector. I wish to stress that applied DC fields have not been rigorously tested at all in the mammalian spinal cord. This latter statement underscores the nature of our work, partially supported by this contract.

C. Approach and Methodology; Annual and Final

1. Measurements of endogenous currents in bone using a vibrating probe.

The vibrating probe was designed to measure the minute ionic currents that traverse a variety of developing single cells, immersed in a natural medium. (20) The electrode itself is a platinum black ball, about 20 μm in diameter. This ball is vibrating (at about 400 Hertz) between two positions (typically 30 μm apart) by means of piezoelectric bender element to which a small voltage is applied. When the electrode is vibrated near a biological current source (or a calibration source) immersed in a natural medium, it measures the minute voltage differences in the plane of its vibration between the extremes of its 30 μm excursion. This is accomplished by using a phase frequency lock-in amplifier to both set the frequency of vibration, and to amplify the signal. These two modalities are then simply tuned together. Since we directly resolve this minute voltage difference and we can easily measure the resistivity of the medium with a conductance bridge, then we calculate the current density entering or leaving the source using an analog of Ohm's Law for extended media: $I = \frac{\nabla}{\rho \Delta}$ where I is the current density in

Amps/cm^2; ∇, the voltage gradient in volts/cm: ρ, the resistivity of the medium in ohms cm; and Δ is the amplitude of vibration. A picroampere source is used to supply a known current by which we calibrate the vibrating probe system. In summary then, the vibrating probe system allows determinations of current density entering or leaving a single cell (or whole tissue) immersed in a natural medium (with a spatial resolution of about 20 to 30 μm). The determinants of current density and potential difference are on the order of 100 to 1000 fold more sensitive than what can be achieved by conventional (static) electrodes.

2. Stimulator Design for Implantation into Animals: Annual 1984-85

Implantable stimulator assemblies were fabricated in the following manner: The voltage source was a lithium manganese dioxide 3 volt unit (Sanyo CR1220). This unit provided 30 milliamp hours capacity and is very small in size (2 mm stack height, 12.5 mm diameter, and 0.8 gm). The battery was connected in series to the rest of the following components by means of silver conductive epoxy: a small fixed resistor, a 3 terminal adjustable constant current source (National Semiconductor, LM-334), and at each pole, a 3-4 mm length of chlorodized silver wire (AgAgCl contacts to the salt bridge). Two millimeters of the AgAgCl contacts were masked off, and the unit is dipped in a bakelite electronics potting compound (stycast 261, Emmerson and Cummings). (This provided structural durability to all electrical connections). A 10 cm long, 1 mm O.D. silastic tube was filled with a mammalian Ringers - agar slurry and a cotton string. Two of these were coiled tightly (but not compressing the tube) and each was attached at one end to the AgAgCl battery connection. The tube is slipped over the AgAgCl wire and glued in place with medical grade elastomer. Within 1-3 minutes this connection was sound and the voltage source and the coiled salt bridges were dipped into medical grade elastomer and stored in zirconium chloride in Ringers. Prior to surgical implantation a long (ca 12-14 cm) wick electrode (silastic tube filled with Ringers and a cotton string) was slipped into the open bore of the salt bridge and the connection electrically and structurally sealed with medical grade elastomer. The unit was then ready to be implanted into the rat or guinea pig periotoneal cavity, the long electrodes can be trimmed to size after they are surgically routed beneath the skin to the exposed spinal cord (see below) or to a femur in bone studies (see below).

3. Stimulator Design: Final Report

The above implantable stimulator served us well during the experiments of last year, however, we are abandoning this design and moving to an external voltage source. This is necessary because we now wish to explore the responses in both bone and nerve to larger field strengths. The implantable unit can only effectively drive about 10 μA total current, resulting in fields on the order of hundreds of mV/mm at the spinal cord (for example). We would like to drive currents on the order of 5 fold this value bringing the fields into the mV/mm range in the hopes of a more luxurious growth of neurons or a more striking response in bone. Thus we have to go to larger voltage sources that are too large to be implanted into the animal. We are now fabricating stimulator assemblies (still using salt bridges, wick electrodes and constant current sources etc.) with 30 volt sources that will sit on the animal's back in a saddle. Guinea pigs tolerate this with no discomfort and do not try to dislodge it. Rats, on the other hand, present a

problem; however, we have found that if the saddle is mounted high on the back near the neck - they can not get to it to remove the assembly.

4. General Surgical Procedures: Spinal Cord Studies

The spinal cord will be exposed by conventional thoracic laminectomy - and a dorsal column transection will be performed using an electolytically sharpened tungsten needle. We wish to study dorsal column transections because: a) their characteristics - both morphological and electrophysiological - are well described in the literature; b) they are accessible and easily transected; c) the anatomy of the dorsal tracts and their segmental roots provide a more focused (and less ambiguous) approach to physiological tests and anatomical techniques (such as HRP filling at severed roots, see below) d) the injury deficit is slight, animal mortality is little affected.

Overall, we feel that the contusion injury model is inappropriate for determining a clear answer to the question we raised in our contract proposal: "Can mammalian CNS neurons respond to an applied field?"

If neurons do respond, a more clinically useful injury model would be another level of investigation - coupled with behavioral tests. However, it is our opinion that a contusion injury was too complex a system with which to begin these studies.

a. Battery and Electrode Implantation

The stimulator system will be joined to the wick electrodes by means of medical grade elastomer a few hours before implantation. The entire unit is soaked in Zirconium Chloride (in Ringers) during this period.

The peritoneal cavity of the animal is opened by ventral incision, and the stimulator unit inserted into the cavity - the long wick electrodes protruding out of the cavity. The peritoneum is surgically closed and the electrodes routed beneath the skin to the dorsal surface of the animal. After the spinal cord is exposed, the wick electrodes are gently secured to the deeper musculature of the back by "loop ties" using sterile suture (without piercing or compressing the silastic tube at these levels).

At this time, and before closing the laminectomies, we insert a small staple shaped device (after Foerster device) into the dorsal column transection. This "marker" is left in place during the experiment. It is carefully removed after the spinal cord is fixed and prior to sectioning. The small holes left in the tissues by the device are excellent for marking the exact boundaries of the lesion - even months post-transection.

b. Morphological Analysis

Spinal cord tissues are dissected after intra cardiac perfusion - fixation. Cross sections of the dorsal column (or pyramidal tract) will be processed and embedded in plastic for sectioning on an LKB Ultratome-III ultramicrotome. 1 m "thick" sections will be stained with Toluidine blue and observed. If an area of interest is found - for example an area

near the original lesion - the block will be reduced by trimming and serial thin sections will be taken for transmission Electron Microscopy. Serial L.M. cross sections will allow a morphometric analysis of axons rostral, caudal to, and within the area of the lesion using a Leitz Videoplan Computer Enhanced Image analysis system available to staff members in this department.

5. HRP Intracellular Marking

Visualization of prepared specimens are accomplished in cross sections of plastic embedded specimens at both the L.M. and E.M. levels, and such techniques are well regarded as providing excellent evidence for structural continuity of axons through lesions. We will apply these techniques principally to the dorsal columns where axons can be filled by immersion in 2% HRP at transections of the columns just prior to sacrifice.

D. Conclusions

1. Bone Bioelectricity: Annual and Final Report

We have completed the long study of endogenous currents traversing intact and lesioned mammalian bone with the Vibrating Probe System (27).

Intact, living mammalian bone (the middle phalanx of the mouse) at physiological temperature drives a strong electric (ionic) current through itself (ranging from 3 to 12 $\mu A/cm^2$ in density). Current preferencially enters the primarily cartilagenous regions (the epiphysis - articular cartilage) and in various places along the shaft. This incurrent is balanced by diffuse out-currents that leave the bone usually in the shaft region (diaphysis), and occasionally at the metaphysis. A fracture to bone produces an enormous leakage of current into the lesion. Initial densities of fracture currents are over 100 $\mu A/cm^2$ and decline with time. By 5 to 30 minutes, a stable "plateau' current of about 5 $\mu A/cm^2$ is reached. This plateau current persists indefinitely.

The early, large, and declining in-currents are independent of cellular metabolism and are produced by a deformation of bone substance. (This component can be observed in dead, glutaraldehyde fixed bone). The steady persistant "plateau" current is, on the other hand, dependent on cellular metabolism, and is produced by an active cellular battery. (It is temperature dependent, is responsive to changes in the ionic composition of the media, and is never observed in dead bone). Overall the geometry of the fracture current loop is complex and highly variable. Current always enters the fracture gap, but may leave the bone almost anywhere in the immediate vicinity of the lesion.

The steady charge flow ("plateau current") driven by a cellular battery, is mainly carried by Na^+ and Cl^-, both ions actively taken up by the bone compartment (the extracellular fluid of bone) by separate ionic pumps from the extracellular fluids of the body. We have not isolated the investment of bone driving this current, but by a process of elimination infer it to be the endosteum, or the sac-like syncytium surrounding the marrow (21). Thus, in mammalian bone, current on the order of 10's of $\mu A/cm^2$ will be driven chronically into a fracture. This current, being of dual origin, will be

maintained in loaded or unloaded bones. We view this two component fracture current to perhaps be a controlling feature in the repair process and worthy of further investigation, and we can make some suggestions concerning the nature and role of currents in bone repair and remodelling.

It is commonly taught that a gradient of "positive" and "negative" potentials line up on either side of an angulated fracture. In fact, no predictable voltage gradient can exist about fractures or along the surfaces of lesioned bone. Current always enters the fracture gap - but can leave almost anywhere in the bone to complete the circuit. This is almost certainly due to the complex and variable geometry of fractures.

Investigators interested in short-lived, load-induced potentials in bone (piezoelectricity or streaming potentials) will also have to accommodate the fact that dead bone - bone material) can produce enormous densities of current that may last 15 minutes. The decay time of piezoelectric signals in bone in physiological media is in microseconds. Thus we can effectively rule out piezoelectricity as a major contributor to this steady current production in bone under physiological conditions. Streaming potentials can be produced for tens of seconds under laboratory conditions. Thus, we lean more toward this mechanism for the production of current in non-living bone, however, it is important to state that at present there exists no theoretical or laboratory model that can accommodate this early flexure current.

The metabolically dependent Na^+ and chloride pump producing the plateau current is a new observation in hard tissue biology. First, Ca^{++}, K^+, carbonates and phosphates, as well as H^+ transport between the extracellular fluid of bone (the bone compartment) and the extracellular fluid of the body have received attention from bone physiologists (28). Our measurements show that Na^+ and Cl^- are actively pumped into (perhaps across) bone investments. The concept of a "bone membrane" responsible for such ion pumping has been debated for years. Histology and ultrastructure of "epitheleoid" tissues of bone (lining cells, periosteium, endosteum) however, do not support this notion (22,23). The investments of bone are physically discontinuous and their cells lack tight junctions (usually necessary for the maintenance of large transepithelial potentials). Not withstanding, I would suggest that there has to be a bone membrane. It apparently operates continually in intact bone, is temperature dependent, and contains specific pumps for Na^+ and Cl^-. It also serves as the pump driving charge into the electrical "leak" of the fracture.

To surgically heal a non-union with applied D.C. currents, 10's of $\mu A/cm^2$ are pulled into the fracture gap with implanted cathodes (the negative electrode). The positive electrode may be subdermal, or even external. A normal fracture likewise drives 10's of $\mu A/cm^2$ into the fracture gap (current entering the lesion). This current (comprised of two components as explained) will be persistent whether the bone is stressed or not. These observations provided a possible rationale behind the success of artificial currents used in clinical treatments (derived by largely empirical means), since the clinical treatments apparently mimic the natural electrophysiology of wounding in bone tissue. This suggests that the common denominator in biological non-union (no matter what disease state leads to their expression) may be a defect in the electrophysiology of fracture repair.

2. Artificial Stimulation of Bone Remodelling: Final Report

We have carried out bone bioelectricity studies another step. This is to test if a chemically pure movement of charge (an electric current) can indeed change the morphology of bone. To our knowledge all clinically oriented studies employ stimulation techniques using metal electrodes that contaminate the target tissue with metallic or metallic salt electrode product contaminants. We decided to test if bone tissue would respond to stimulation via wick electrodes (which carry current to the tissues through a solution of electrolytes similar to body fluids and is free of electrode production contamination). Using the same stimulators described above, we stimulated intact femurs of large (400 gram) rats for 6-weeks and then analyzed the bone morphology histologically and morphometrically. (Surgery was identical to that described for the guinea pig spinal cord experiments except that the electrodes were routed to the hind limb and surgically fastened to the intact femur about 1 mm.) Dead batteries implanted in a similar fashion provided the sham-treated control series. In controls there was some remodelling due to the periosteal reaction of bone to the physical irritation of the silk tie securing the electrodes nearby. In these experiments the entire profile of the bone was altered. Bones were grossly altered in shape, sometimes displaying large protuberances beneath the stimulation electrodes. Sometimes the diaphysis was abnormally thinned producing a "dumb bell" shaped bone. Interestingly, small (ca. 1 mm base to apex) protuberances were found in the endosteal surface of the bones within the marrow cavity. These were always located adjacent the stimulation electrodes on the far side of the marrow cavity. All together we are able to document gross changes in shape in bone and abnormal points of deposition and resorption correlating with the position of the electrodes. These data are being prepared for submission to the Journal of Orthopedic Research.

3. Field Effects on Mammalian Spinal Cord Neurons in Vivo: Annual and Final

There have been few reported attempts at influencing axonal development in either the peripheral or the central nervous system of mammals using applied DC electric fields. In fact, I know of no rigorous published attempt at influencing CNS regeneration (particularly spinal cord) with applied fields, perhaps because this model system is so complex in the mammal. In particular, certain problems have plagued spinal cord regeneration studies for years and have rendered many if not most of these experiments ambiguous. The two major pitfalls have traditionally been 1) marking the exact site of the lesion and 2) developing a means to precisely identify axons within the cord to know their origin and trajectories after injury. The first problem is a great one because of the gross pathological responses of cord tissue to compression or cut. After two months or most post injury, an area of many mm's on either side of the focus of damage becomes scarified. The precise location of the original plane of damage is therefore largely unknown. If one were to induce regeneration of axons for a few mm's, this growth (in relation to the plane of the lesion) would go undetected since it could not easily be indexed. The second stumbling block to analyzing the responses of severed or damaged axons in the spinal cord of mammals is the lack of a precise way to histologically observe axonal regeneration or the lack of it. Older staining techniques allowed visualization of impressive numbers of axons - however - one cannot usually delineate originally undamaged (spared) axons from transected ones. Additionally, one cannot usually determine if axons are long tract, local, or sympathetic since all are stained equally;

i.e., if axons were observed within a lesion, they could be fibers that were spared, they could be segmental axons lying near the plane of transection, or even sympathetic axons emerging from interrupted blood vessels (sympathetic fibers are known to grow well in the environment of the spinal cord). Using an intracellular marker such as HRP and electron microscopy, one could trace individual axons laboriously, but such procedures provide only a tiny window on the overall neuronal response to injury.

Our laboratory has recently reach a solution to testing the effects of imposed electric fields across cord transections in large fully adult guinea pigs. We have adopted and applied A. Foerster's (24) marking device to precisely identify the exact boundaries of a hemisection. This device is placed into the fresh transection and is allowed to stay in place during the duration of the experiment. It is removed after the cord is fixed prior to sectioning. The tiny holes left in horizontal longitudinal sections are effective markers for the boundaries of the lesion. To visualize axonal regeneration, we wait one day prior to the time of sacrifice and insert crystals of HRP into a shallow transverse incision of the cord 2-3 vertebral segments caudal to the original hemisection. This laminectomy is closed and the animal is allowed free movement until the next day when it is sacrificed. We exclusively concentrate on axons of the dorsal columns, which intensely fill with HRP during this 24-hour uptake time. Since HRP is inserted caudal to the original experimental lesion, we only visualize long tract axons of this tract. Intact fibers of the tract or any other fibers more rostral to the fill point are simply not stained. Furthermore, any filled fibers found rostral to the lesion had to have regenerated there since only axons well caudal to the lesion would have taken up the HRP. Combining the marking device (delineating the boundaries of the lesion), natural markers (such as the severed central canal), and the staining of a subpopulation of long tract dorsal column neurons allowed us to precisely identify the fibers originally severed and their positions within the cord before and after injury.

Implantable stimulators (delivering 1, 5, and 10 μA total current) were surgically inserted into the peritoneal cavity in three groups of adult guinea pigs. The stimulating electrodes were routed beneath the skin to the back where they were fastened within the vertebral column next to (but not touching) the exposed spinal cord. These two electrodes were situated about 2 cm either side of a transverse transection of the dorsal spinal cord (down to the level of the central canal). The animals were sacrificed 50-90 days later and current-treated animals were analyzed and compared with the sham-treated controls. Since all known neuronal responses to electricity are mediated by distally negative fields, the negative electrode was positioned rostral to the lesion. (The dorsal columns being ascending pathways). In all untreated animals (simple transections) and sham-treated controls (implanted with dead batteries and electrodes), there were some signs of endogenous regeneration. The clarity of the 75 μm thick sections containing darkly filled axons provided us with extraordinary cytological detail. We were able to visualize growth cone terminals on axons, bifurcations of large bore myelinate axons within the dorsal column pallisade, and axon deviations around obstacles such as capillaries. This regeneration was not sufficient, however, to bring the terminals of these axons even close to the glial scar that spread around the lesion some 1 mm more rostral. (Axons first dieback some 1-2 mm caudally before beginning to regenerate). These observations of regeneration within untreated cords are somewhat novel, however, the total growth associated with this regeneration is scant. Overall, our observations

are generally consistent with what is described in literature (25,26). In all current-treated animals, many axons were found to have grown well into the scar. Once reaching the boundary of the glial scar, fibers twisted and turned and followed atypical pathways through this dense cellular mass. In the 10 μA series, axons were also found to have traversed the lesion from the caudal to the rostral segment of the spinal cord. The pathway that these dorsal column axons chose to follow depended upon their position within the pallisade. Lateral axons deviated toward the lateral margin of the cord once entering the scar and grew around the edge of the lesion through uninjured cord parenchyma. Fibers lying central within the dorsal columns grew toward the exact plane of transection, and then deviated ventrally, growing around the ventral boundary of the lesion (as delineated by our marker device), passed beneath the swollen ends of the severed central canal, and ascended the rostral face of the lesion before projecting rostrally toward the brain. The ability of these axons to traverse such foreign terrain, navigate about obstacles (such as the marker device and the segments of the central canal) and finally project back to a roughly appropriate position within the spinal cord was striking. Altogether we were able to determine that indeed central axons in the mammal will respond to an applied field. We do not know if a return of function accompanies this regeneration, but are designing experiment to test this.

The ability of fibers to grow around obstacles, traverse foreign terrain, and reach their respective positions in the rostral segment of the cord suggests that a general developmental pageant was awakened by the imposition of the field. The responses we observed are more complicated than a simple stimulation of outgrowth or directional effect imposed by the field. For example, the long axis of the field within the guinea pig spinal cord was parallel with the long axis of the cord. Fibers deviated markedly from this vector while growing from the caudal to rostral segment. It did not appear that these fibers were simply vectoring on the cathode, rather, they were pathfinding. Robinson's group (13) found that when neuroblasts were dissegragated and placed in culture, an induction of neurite outgrowth occurred. This induction of development was more profound in cultures with an imposed voltage gradient. As previously suggested, a steady electrical field might be a normal component of the extracellular environment about developing nervous tissue. When an axon is severed and separated from its target, a similar field may trigger a cascade of developmental mechanisms that may not only lead to an induction of growth but to target seeking and the formation of connections as well.

E. Recommendations; Annual and Final Report

Bone Bioelectricity

It is clear that a strong steady current is driven through fractures in mammalian bone. This current is similar in magnitude and polarity to the currents that have been found to be effective in promoting the healing of chronic non-unions. We would suggest that further research into these natural currents and their role in repair and remodeling will lead to an expanded role for clinically imposed currents. For example, we envision the possibility of accelerating the repair of normal and healing fractures. We

also envision the use of a hand-held vibrating probe in the surgical arena. Such a device, when placed into an exposed and debreded fracture gap may register an electrical profile that could allow the orthopedic surgeon to prognosticate the likelihood or rate of repair. Understanding more of the endogenous currents in bone may also open a new understanding of bone physiology and how hard tissue mediates the ionic homeostasis of the body.

Spinal Cord Regeneration

The ability to induce growth in the lesioned spinal cord opens numerous clinical possibilities, not all of which are restricted to the cord. The induction of substantial neuronal regeneration in brain tissue may lead to novel clinical approaches in dealing with head trauma as well as other debilitating traumatic lesions to any part of the CNS axis.

We are now testing if the induced regeneration we have observed in dorsal columns will be commensurate with functional recovery. This will be investigated in motor columns as well. Affirmative answers to these experimentals will probably lead to clinical trials.

REFERENCES

1. Brighton, C.T. 1981. "The treatment of non-unions with electricity." J. Bone and Joint Surgery 63A:847-851.

2. Deafler, S.F. 1981. "Electrical phenomena associated with bones and fractures and the therapeutic use of electricity in fracture healing." J. Med. Eng. Technol. 5:73-79.

3. Pienkowski, D., and S.R. Pollack. 1983. "The origin of stress generated potentials in fluid-saturated bone." J. of Orthopaed. Res. 1:30-41.

4. Gross, D., and W.S. Williams. 1982. "Streaming potentials and the electromechanical response of physiologically moist bone." J. Biomechanics 15:277-295.

5. Friedenberg, Z.B., and C.T. Brighton. 1966. "Bioelectric potentials in bone." J. Bone and Joint Surgery 48A:915-923.

6. Friedenberg, Z.B., and H.G. Smith. 1969. "Electric potentials in intact and fractured tibia." Clin. Orthop. 63:222-225.

7. Borgens, R.B. 1982. "What is the role of naturally produced electric current in vertebrate regeneration and healing?" Int. Rev. Cytol. 76:245-298.

8. Weiss, A.B., J.R. Parsons, and H. Alexander. 1980. "Direct current electrical stimulation of bone growth: Review and current status." J. Med. Soc. N.J. 77:523-526.

9. Ingvar, S. 1920. "Reaction of cells to galvanic current in tissue culture." Proc. Soc. Exp. Biol. Med. 17:198-199.

10. Jaffe, L.F., and M.M. Poo. 1979. "Neurites grow faster toward the cathode than the anode in a steady field." J. Exp. Zool. 209:115-127.

11. Marsh, G., and H.W. Beams. 1946. "In vitro control of growing chick nerve fibers by applied electric currents." J. Cell Comp. Physiol. 27:139-157.

12. Weiss, P. 1934. "In-vitro experiments on the factors determining the course of the outgrowing nerve fiber." J. Exp. Zool. 68:393-448.

13. Hinkle, L., C.D. McCraig, and K.R. Robinson. 1981. "The direction of growth of differentiating neurons and myoblasts from frog embryos in an applied electric field." J. Physiol. 314:121-135.

14. Patel, N., and M.M. Poo. 1982. "Orientation of neurite growth by extracellular electric fields." J. Neurosci. 2:483-496.

15. Patel, N.B., and M.M. Poo. 1984. "Perturbation of the direction of neurite growth by pulsed and focal electric fields." J. Neurosci. 4:2939-2947.

16. Borgens, R.B., J.W. Vanable, Jr., and L.F. Jaffe. 1977. "Bioelectricity and regeneration I: Initiation of frog limb regeneration by minute currents. J. Exp. Zool. 200:403-416.

17. Borgens, R.B., J.W. Vanable, Jr., and L.F.. Jaffe. 1979. "Small artificial currents enhance Xenopus limb regeneration. J. Exp. Zool. 200:217-255.

18. Borgens, R.B., J.W. Vanable, Jr., and L.F. Jaffe., 1979. "Reduction of sodium dependent stump currents disturbs urodele limb regeneration." J. Exp. Zool. 209:377-386.

19. Borgens, R.B., E. Roederer, and M.J. Cohen. 1981. "Enhanced spinal cord regeneration in lamprey by applied electric fields." Science 213:611-617.

20. Jaffe, L.F., and R. Nuccitelli. 1974. "An ultrasensitive vibrating probe for measuring steady extracellular currents." J. Cell Biol. 63:614-628.

21. Menton, D.N., D.J. Simmons, B.Y. Orr, and S.B. Plurad. 1982. "A cellular investment of bone marrows." Anat. Rec. 203:157-164.

22. Neuman, W.F. 1980. "Bone mineral and calcification mechanisms." In: Fundamental and Clinical Bone Physiology, ed. M.R. Urist, pp. 83-107. Philadelphia: J.B. Lippincott Co.

23. Matthews, J.L. 1980. "Bone structure and ultrastructure." In: Fundamental and Clinical Bone Physiology, ed. M.R. Urist, pp. 4-44. Philadelphia: J.B. Lippincott Co.

24. Foerster, A.P. 1982. "Spontaneous regeneration of cut axons in adult rat brain." J. Comp. Neurol. 210:335-356.

25. Kiernan, J.A. 1979. "Hypotheses concerned with axonal regeneration in the mammalian nervous system." Biol. Rev. 54:153-197.

26. Berry, M. 1979. "Regeneration in the central nervous system." In: Recent Advances in Neuropathology, ed. W. T. Smith, and J.B. Cavanaugh, pp. 67-111. New York: Churchill Livingstone.

27. Borgens, R.B. 1984. "Endogenous ionic currents traverse intact and damaged bone." Science 225:478-482.

28. Borgens, R.B., J.W. Vanable, Jr., and L.F. Jaffe. 1979. "Bioelectricity and regeneration." Bioscience 29:468-474.

29. Roederer, E., N.H. Goldberg, and M.J. Cohen. 1983. "Modification of retrograde degeneration in transected spinal axons of the lamprey by applied DC current." J. Neurosci. 3:163-160.

GLOSSARY

Dorsal Columns These large spinal cord tracts are bundles of neurons that project into the spinal cord from segmental ganglia lying just outside the cord itself. Sensory information (largely) is carried to the brain by these tracts that ascend the cord.

Laminectomy Surgical exposure of the spinal cord within the vertebral column.

Neurite A general and non-specific term for a neuronal process.

Wick electrode An aqueous "wire". Stimulating electrodes fashioned from a silastic tube, filled with mammalian Ringers and a cotton string (the "wick"). Thus current is carried to the tissues by a conductive solution similar to body fluids and not by metallic wires (which contaminate the tissues with electrolysis products). The wick ensures that there is electrical continuity in case air bubbles form and partially occlude the inner diameter of the tube.

Orthodromic and Antidromic stimulation and recording Experimentally evoked Action Potentials whose conduction pathway is in the same direction as natural conduction are orthodromically stimulated. For example: orthodromic stimulation of a motor neuron would involve stimulating near the soma (or ganglion) and recording at the periphery. Antidromic stimulation and recording would be the reverse of this regimen.

DISTRIBUTION LIST

4 copies	Commander Letterman Army Institute of Research (LAIR), Bldg. 1110 ATTN: SGRD-ULZ-RC Presidio of San Francisco, CA 94129-6815
1 copy	Commander US Army Medical Research and Development Command ATTN: SGRD-RMI-S Fort Detrick, Frederick, Maryland 21701-5012
2 copies	Defense Technical Information Center (DTIC) ATTN: DTIC-DDAC Cameron Station Alexandria, VA 22304-6145
1 copy	Dean School of Medicine Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, MD 20814-4799
1 copy	Commandant Academy of Health Sciences, US Army ATTN: AHS-CDM Fort Sam Houston, TX 78234-6100


DATE
FILMED
5-8